

# Preparation and characterization of hydrogel-based delivery systems for bioactive molecules targeting transthyretin cardiac amyloidosis

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**Abstract.** Cardiac amyloidosis is a disorder characterized by the misfolding and aggregation of proteins such as transthyretin (TTR), presenting significant therapeutic challenges. Effective treatment requires the controlled and sustained delivery of biologically active molecules to affected tissues. Flavonoids such as quercetin, curcumin, and mangiferin have shown the ability to stabilize TTR tetramers, thereby inhibiting amyloid fibril formation. Despite their promising therapeutic potential and favorable preclinical results, the clinical translation of flavonoids has been limited by poor bioavailability, rapid metabolism, and chemical instability. To overcome these limitations, tunable hydrogel delivery systems have emerged as a viable approach to enhance flavonoid bioavailability. Consequently, a controlled-release chitosan-based hydrogel drug delivery system was developed. The surface morphology of the synthesized hydrogels was examined using scanning electron microscopy, while their structural integrity and pH-responsive properties were evaluated through gel fraction and pH-dependent swelling studies respectively.

## 1 Introduction

Cardiac amyloidosis (CA), driven primarily by transthyretin (TTR) misfolding and fibril deposition in the myocardium, leads to restrictive cardiomyopathy and heart failure [1-4]. The pathophysiology involves TTR tetramer dissociation into misfolded monomers that aggregate into toxic oligomers and fibrils, complicating therapeutic intervention. While current approaches, such as TTR stabilizers and gene-silencing therapies, have shown improved outcomes [5-12], limitations in targeted delivery, long-term efficacy, and accessibility highlight the need for novel strategies.

Natural biological molecules, particularly flavonoids such as quercetin, curcumin, and mangiferin, present a promising therapeutic avenue. These polyphenols have demonstrated a robust ability to stabilize TTR tetramers in preclinical studies, inhibiting the initial conformation changes driving cardiac amyloidosis [13-15]. Their favorable safety profile and

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multi-target potential further underscore their candidacy. However, a wide translational gap exists between their preclinical efficacy and clinical success, primarily due to poor solubility, low permeability, rapid metabolism, and instability [16,17].

To address these limitations, advanced drug delivery systems are crucial. Hydrogels-based systems, with their tunable three-dimensional networks, provide a viable approach to encapsulate flavonoids, protect them from degradation, and control their release kinetics. Hence, enhancing the bioavailability and therapeutic potential [18]. However, the successful implementation, requires optimization of parameters such as crosslinking density, polymer composition, and surface properties to ensure biocompatibility and efficacy..

In this study, we report the design and development of a novel chitosan-based hydrogel delivery system for flavonoids. By investigating key formulation parameters such as crosslinking density, polymer matrix composition, and pore architecture, we aim to develop a controlled-release system that could enhance the bioavailability and efficacy of natural TTR inhibitors..

## **2 Methodology**

### **2.1 Materials**

The chitosan used weighed 200 kDa (deacetylation degree 83 %) and was bought from Bioprogress LLC, Russia. Pullulan was supplied by Tokyo Chemical Industry Co., Ltd. The other chemicals were sodium periodate (95% purity, NevaReactiv), sodium hydroxide (95% purity, NevaReactiv), glacial acetic acid (99.8%) ethylene glycol, phosphate buffer tablets (pH 7.4) and hydrochloric acid were of analytic grade.

### **2.2 Chemical oxidation of pullulan**

Chemical modification and purification of pullulan to aldehyde pullulan were conducted using the approach described by Elangwe et al. [19].

### **2.3 Dynamic viscosity of precursor polymer solutions**

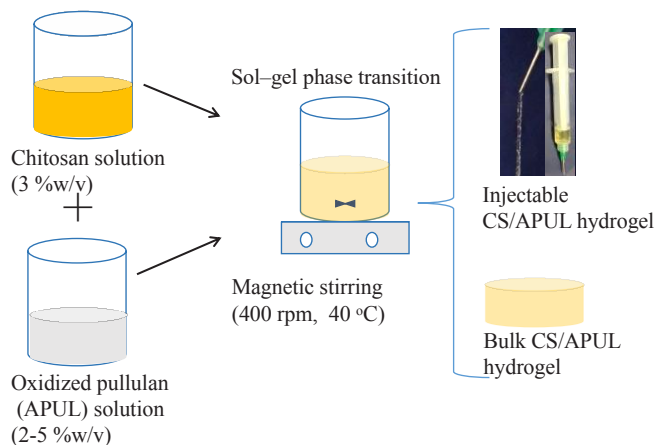
Rheological studies are integral to the development of drug delivery systems. Consequently, the dynamic viscosity of aqueous polymer solutions was investigated. The analysis, performed with an Anton-Paar MCR 502 rheometer (Austria), included chitosan at 3% w/v, pullulan at 5% w/v, and oxidized pullulan at concentrations of 2, 3, 4, and 5% w/v.

### **2.4 Preparation of hydrogel systems**

The initial step in the synthesis of the chitosan-based hydrogel involved preparing a 3% w/v chitosan solution. Chitosan powder (1.5 g) was dissolved in 50 mL of 1.0% aqueous acetic acid, with continuous stirring maintained at 50°C for 4 h to obtain homogeneity. Aldehyde pullulan (APUL) solutions (2-5% w/v) were prepared at room temperature. For gelation, aldehyde pullulan solutions were mixed with the chitosan solution in a 1:1 volumetric ratio at 40 °C under magnetic stirring, and the obtained hydrogels were aged for 24 h at room temperature (Fig. 1).

The hydrogel network structure results from synergistic interactions. The primary covalent crosslinks are formed through a Schiff base reaction between the aldehyde groups (-CHO) of oxidized pullulan and the primary amine groups (NH<sub>2</sub>) present in chitosan. The hydrogel is further reinforced by secondary interactions such as hydrogen bonds and physical

interlocking of entangled chitosan and pullulan chains. Fig. 1 shows the preparation steps of CS/APUL hydrogel.



**Fig. 1.** Preparation of chitosan/oxidized pullulan hydrogels.

## 2.5 Determination of gel fraction

The gel fraction or content, a quantitative measure of crosslinking degree, was determined to evaluate the crosslinking efficiency of the hydrogel network formation. This parameter represents the percentage of polymer incorporated into the insoluble, crosslinked matrix. Pre-defined hydrogel samples (~20 mg, n=3) were immersed in distilled water at 37°C for 48 h to extract the soluble, unreacted fraction. When the samples attained equilibrium swelling, the insoluble gel fraction was dried to a constant weight. The gel fraction was determined as the mass ratio of the final insoluble portion to the initial dry sample, according to Equation (1) [20].

$$\text{Gel fraction (\%)} = (W_d / W_0) \times 100 \quad (1)$$

where  $W_d$  and  $W_0$  are the dried weight of the hydrogel and dried weight of the gelled part after extraction, respectively.

## 2.6 pH-responsive property

To determine the pH-sensitive property of the chitosan/aldehyde pullulan hydrogels, their dimensional change was monitored. Hydrogels in cylindrical form were developed, and their diameter was measured. These samples were then incubated in phosphate buffer solutions at varying pH (5.5, 7.4, and 8.5). The change in diameter was recorded over time, with measurements taken at 1, 3, and 24 h intervals. The extent of shrinkage was expressed as a ratio calculated from the initial and final diameters according to Equation (2) [21].

$$\text{Diameter shrinkage ratio (\%)} = (L_0 - L_1 / L_0) \times 100 \quad (2)$$

where  $L_0$  and  $L_1$  denote the diameter of the C/APUL hydrogel before and after being incubated in phosphate buffer solutions, respectively.

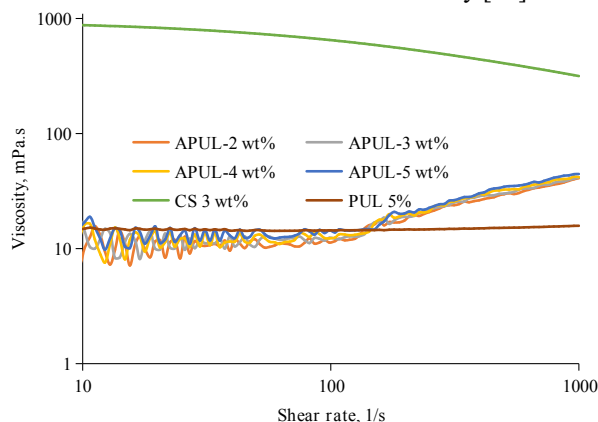
## 2.7 Surface Morphology

The internal morphology of CS/APUL hydrogels was examined by scanning electron microscopy. The hydrogel samples were lyophilized for 72 h, sectioned with a blade in order to reveal their pore architecture, and coated with gold. Imaging was performed on a JEOL JSM-7001F microscope at 5 kV, with magnifications of 100  $\mu\text{m}$ .

## 3 Results and Discussion

### 3.1 Dynamic viscosity of chitosan and pullulan solutions

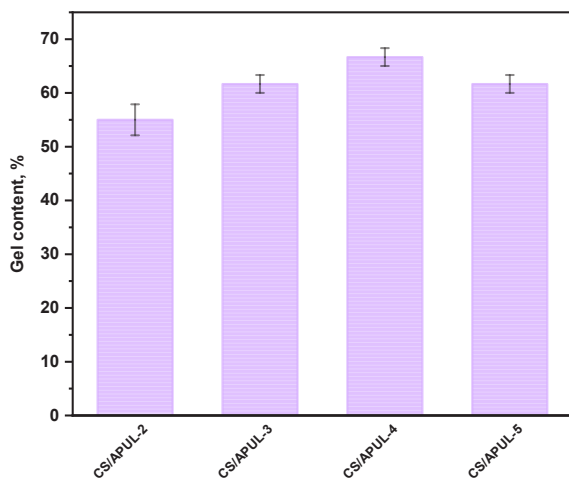
The shear-thinning behavior of chitosan is driven by its ionized amine groups, as shown in Fig. 1, with viscosity decreasing as the shear rate rises. This effect is attributed to the exposure of  $-\text{NH}_3^+$  groups, which modulates electrostatic and steric repulsion and leads to rearrangement of the polymer network. Conversely, aldehyde-functionalized pullulan demonstrated shear-thickening owing to aldehyde moieties that form inter- and intramolecular hemiacetal bonds with unreacted hydroxyl groups within the polymer. Brady's cluster formation mechanism, supported by Stokesian dynamics experiments, provides a plausible explanation for shear thickening, wherein shear-induced micro-assemblies increase the fluid's viscosity [22]. It is established that aldehyde groups introduced through chemical modification can form hemiacetal bonds with hydroxyl groups. In aldehyde pullulan solutions, shows a shear-thickening behavior, where viscosity increases markedly at shear rate surpasses approximately 100  $\text{s}^{-1}$ . This shear-induced crosslinking, which generates both intra- and intermolecular hemiacetal and hemialdal bonds, is responsible for the observed increase in viscosity [23].



**Fig. 2.** Dynamic viscosity versus shear rate of chitosan and pullulan solutions.

### 3.2 Gel content

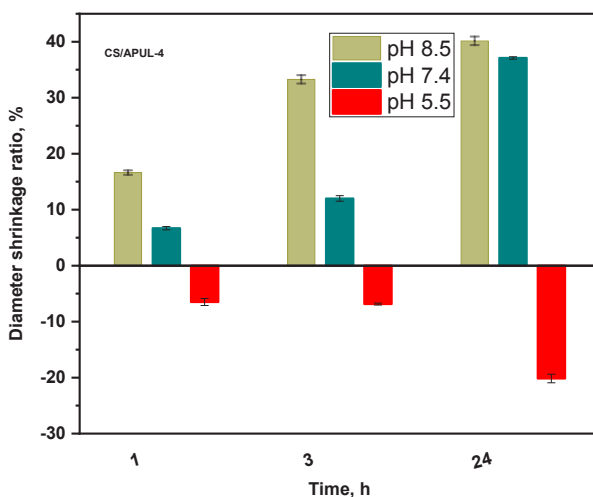
The gel content or fraction, defined as the insoluble polymer fraction, provides a direct measure of the degree of crosslinking and network stability [20]. A higher gel content generally correlates with greater crosslinking density and a more robust three-dimensional network capable of retaining its structural form in aqueous environment. An increasing trend from CS/APUL-2 to CS/APUL-4 (Fig. 3) corresponds to improved crosslinking density. The subsequent decrease for CS/APUL-5 is attributed to unreacted functional aldehyde and hydroxyl groups from higher pullulan content, which may limit further covalent bond formation.



**Fig. 3.** Gel content of chitosan/aldehyde pullulan hydrogel samples.

### 3.3 pH-sensitive behavior

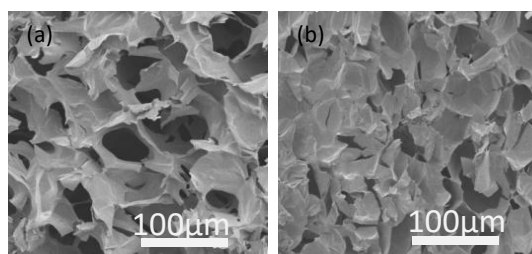
The pH-sensitive behavior of CS/APUL hydrogels was analyzed by immersing the samples in PBS at pH 5.5, 7.4, and 8.5 (37°C for 24 h). Diameter shrinkage was measured at 1, 3, and 24 h intervals. As shown in Fig. 4, hydrogels contracted at pH 7.4 and 8.4, with shrinkage increasing over time and higher alkalinity., confirming a pronounced pH-responsive property. In contrast, swelling occurred at pH 5.5 due to amino group protonation ( $\text{NH}_3^+$ ), which induced electrostatic repulsion, chain expansion, and imine bond weakening. Under alkaline conditions, deprotonation stabilized the imine bonds ( $-\text{C}=\text{N}$ ). This intrinsic pH-dependence is essential for intelligent drug delivery systems, as it allows controlled release of bioactive molecules such as flavonoids in the treatment of amyloidosis, while reducing side effects.



**Fig. 4.** pH-sensitive behavior of CS/APUL hydrogel.

### 3.4 Morphology

Scanning electron microscopy (SEM) analysis of selected hydrogels revealed an open and interconnected porous architecture as shown in Fig. 5. This morphology is advantageous, as it provides hydrogel scaffolds capable of facilitating the encapsulation of natural inhibitors within the chitosan/aldehyde pullulan hydrogel matrix. In addition, such tunable pore architecture is an important feature for enabling the release of payload in a controlled and sustained manner.

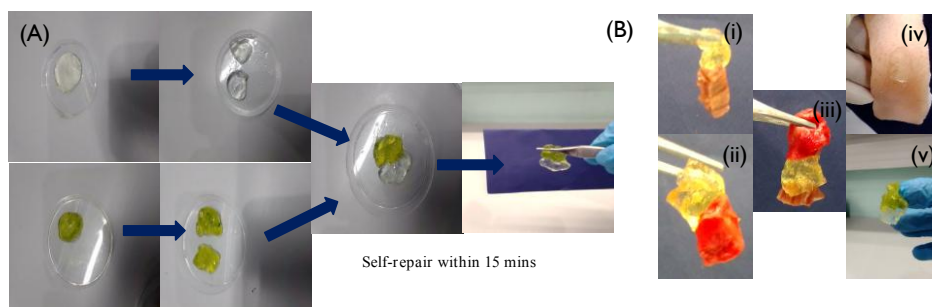


**Fig. 5.** SEM images of (a) CS/APUL-3, (b) CS/APUL-4 hydrogels.

### 3.5 Self-recovery and adhesive properties

The self-recovery ability of chitosan/aldehyde pullulan hydrogels due to dynamic covalent imine bonds, allow remarkable adaptability and longevity [24,25]. The CS/APUL hydrogels demonstrated rapid macroscopic self-recovery, with cut interfaces rejoining in 15 minutes. This behavior is due to dynamic covalent imine bonds ( $-C=N$ ) between the amino groups ( $NH_2$ ) of chitosan and the aldehyde groups ( $-CHO$ ) of oxidized pullulan, alongside supporting hydrogen bonds. Consequently, the hydrogels retained their structural integrity when stretched, as visually confirmed in Fig 6A.

The adhesive properties of CS/APUL hydrogels are governed by synergistic network of both physical and chemical interactions. The base polymer, pullulan, provides inherent stickiness due to its multiple OH groups. When combined with chitosan, the adhesive strength on tissues (such as pig skin, heart) is significantly enhanced through three main mechanisms: hydrogen bonding, imine linkages, and electrostatic interactions. The latter is particularly strong due to the attraction between the chitosan's amino groups and the phospholipids in cell membranes [26], as visually confirmed by adhesion tests on various pig organs (Fig. 6B).



**Fig. 6.** (A) Self-healing behavior of chitosan/oxidized pullulan hydrogel, (B) Demonstrates the tissue adhesion of the hydrogels to pig (i) heart, (ii) lung, (iii) sandwiched between lung and heart, (iv) skin, (v) gloves.

## 4 Conclusion

The adhesive chitosan/oxidized pullulan hydrogel properties such as degree of crosslinking, pore architecture, and pH-responsiveness have been studied. The chitosan/oxidized pullulan hydrogel systems with tunable crosslinked network structure could be potent candidate for targeted administration of bioactive molecules which could yield reduced overdosing requirements, improved biodistribution, and diminished adverse effects, while also lowering overall treatment costs. We plan to investigate the release profile of flavonoids such as quercetin, mangiferin and curcumin for the management of TTR amyloidosis in our future work.

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